Predominant Expression of Fas (CD95) Ligand in Metastatic Melanoma Revealed by Longitudinal Analysis

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The expression of Fas ligand has recently been proposed as a novel tumor escape mechanism for melanoma. To establish the characteristics of Fas ligand expression during the course of melanoma progression we performed a longitudinal study analyzing primary tumors as well as subsequently evolving metastases. In primary melanoma Fas ligand was expressed in two of 20 lesions; this expression was weak and restricted to few parts of the tumors. The Fas ligand positive primary melanomas were rather thick, i.e., 8.5 and 3.8 mm, *versus* a median of 2.4 mm of the remaining tumors. In contrast, for metastatic melanoma Fas ligand expression was present in six of 11 cases investigated. The metastases of primary tumors displaying Fas ligand maintained its expression. As Fas ligand positive melanoma cells are capable of inducing apoptosis in susceptible cells, e.g., Fas positive tumor infiltrating lymphocytes, we tested for the presence of apoptotic cells *in situ* by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling. This analysis revealed that apoptotic cells were present within the Fas ligand positive tumors. The number of apoptotic cells, however, never exceeded 5% of the total cells. Thus, Fas ligand mediated apoptosis does not seem to be a major immune escape mechanism for melanoma but its expression correlates with the stage of melanoma. *Key words: apoptosis/immune escape mechanism/tumor progression. J Invest Dermatol 112:899–902, 1999*

The Fas protein (CD95) is a cell surface receptor that upon cross-linking transduces apoptotic signals in susceptible cells. The corresponding ligand, Fas ligand (FasL), is a type II membrane protein of the tumor necrosis factor family. FasL acts upon direct cell contact (Nagata, 1994). Soluble FasL counteracts the effects of the cellular molecule (Suda *et al*, 1997; Tanaka *et al*, 1998). Fas is expressed in a variety of cells of lymphoid and nonlymphoid origin, as well as in many malignant cell lines; in contrast, FasL expression in normal tissues is restricted to activated T cells, natural killer cells, and macrophage lineages (Nagata, 1994; Suda *et al*, 1995; Griffith *et al*, 1996). Principally, the Fas/FasL system is thought to be involved in the regulation of homeostasis of the immune system (Lynch *et al*, 1995; Griffith *et al*, 1996). Moreover, it is involved in the maintenance of immune tolerance in the anterior chamber of the eye, the testis, and placenta, contributing to the immune-privileged status of these tissues (Griffith *et al*, 1996; Uckan *et al*, 1997).

There is increasing evidence that the Fas/FasL system is involved in tumor-mediated immune suppression. Implications have been found in some hematologic malignancies (Tanaka *et al*, 1996), gastrointestinal tumors (Strand *et al*, 1996; Benett *et al*, 1998), lung carcinoma (Niehans *et al*, 1997), primary cerebral tumors (Gratas *et al*, 1997), and ovarian carcinoma (Rabinowich *et al*, 1998). Furthermore, Hahne *et al* (1996) reported that the FasL protein is present in human melanoma, which may promote the evasion of antitumor immune responses because *in situ* assays revealed that the infiltrating leukocytes underwent apoptosis at a high rate.

As FasL is absent in normal melanocytes, it should be upregulated during tumorigenesis and melanoma progression (Hahne *et al*, 1996). Herein we characterize FasL expression both in primary melanoma as well as in subsequent metastases of these primaries in a longitudinal study.

MATERIALS AND METHODS

Tumor samples Fresh tumor samples from 20 primary and 11 subsequently evolving metastatic melanomas as well as 17 metastatic tumors with no available primary lesions were snap frozen in liquid nitrogen immediately after surgery and stored at –80°C. All metastatic lesions were cutaneous metastases.

Immunohistology Frozen sections were fixed in cold acetone for 10 min followed by removal of endogenous peroxidase with 0.03% H_2O_2 and blocking of nonspecific binding with 10% species-specific serum in 1% bovine serum albumin/phosphate-buffered saline. For FasL analysis, the monoclonal anti-FasL antibody G247-4 (Pharmingen, San Diego, CA) was used. To identify tumor infiltrating T cells an anti-CD3 antibody (Dianova, Hamburg, Germany) was used on serial sections. Antibodies with identical isotypes reacting with irrelevant epitopes served as negative controls. Antibodies were overlaid on to serial sections at a dilution of 1:500 and slides were incubated in a humid chamber for 30 min. With phosphate-buffered saline washes between every step, a biotinylated link antibody was applied for 10 min followed by peroxidase linked to streptavidin for 10 min. After another wash 3-amino-9-ethylcarbazol and H_2O_2 was added (AEC substrate kit, DAKO, Hamburg, Germany) and the slides were incubated in the dark for 10 min. Counter staining was performed with hematoxylin.

***In situ* apoptosis detection** Frozen sections of metastases were acetone-fixed and apoptotic cells were detected using the terminal deoxynucleotidyl

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Table I. Expression of FasL in primary and subsequently evolving metastatic melanoma and detection of apoptotic cells

Age (y)	Gender	Primary tumors Breslow thickness (mm)	FasL expression		Metastases FasL expression		CD3 staining[c]	Apoptotic cells[d]
			Frequency[a]	Intensity[b]	Frequency[a]	Intensity[b]		
53	male	8.6	Ø		Ø	++	+	
71	male	8.5	+	+	++	++	++	+
39	female	6.2	Ø					
69	male	4.0	Ø		+	+	++	+
69	male	3.8	++	+	+++	+++	+++	+
68	female	3.6	Ø					
68	female	3.3	Ø		Ø		+	Ø
51	male	3.1	Ø		++	++	++	+
68	male	2.8	Ø					
45	male	2.7	Ø		Ø		++	Ø
59	female	2.5	Ø		+	++	+	+
75	male	2.3	Ø		Ø	+		Ø
44	male	2.2	Ø					
48	female	2.2	Ø					
57	female	2.1	Ø		++	+	++	+
30	female	2.0	Ø		Ø		+	Ø
68	male	2.0	Ø					
67	female	1.9	Ø					
62	male	1.9	Ø					
40	female	1.8	Ø					

[a]Ø, no; +, less than 5%; ++, between 5 and 50%; +++, more than 50% positive cells.
[b]+, dim; ++, intermediate; +++, bright.
[c]Grading of $CD3^+$ melanoma infiltrating cells (+, absent; ++, non-brisk; +++, brisk; according to Clemente *et al*, 1996).
[d]Apoptotic cells were detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (Ø, no; +, less than 5%).

transferase-mediated dUTP nick end labeling assay; cells containing fragmented single chain DNA are labeled with digoxigenin-11-dUTP and subsequently stained by immunoperoxidase. The assay was performed according to the manufacturer's directions (Oncor, Heidelberg, Germany). Negative controls were made by omitting the terminal deoxynucleotidyl transferase enzyme on representative slides while DNase I treated slides served as positive controls.

RESULTS

FasL is predominantly expressed on metastatic melanoma Although expression of FasL has been described for most metastatic melanoma, detected both *in vitro* and *in situ*, it is not expressed on normal melanocytes. In order to test whether FasL expression is upregulated during melanoma progression, we performed a longitudinal study using primary tumors and subsequently evolving metastatic lesions. Patients' characteristics are given in **Table I**. FasL expression was present in two primary tumors and six metastatic lesions; hence, the percentage of positive lesions increased from 10 to 55% during melanoma progression (**Table I**). The expression of FasL in primary tumors was not homogeneous but restricted to few parts of the tumors suggesting a clonal expansion of $FasL^+$ melanoma cells (**Fig 1*a***). The two primary tumors displaying FasL expression were rather thick, i.e., 3.8 and 8.5 mm of tumor thickness, *versus* median of 2.4 mm for the $FasL^-$ melanomas. Furthermore, both $FasL^+$ primaries gave rise to subsequent metastasis which not only maintained FasL expression but showed an even more intense staining (**Fig 1*b***). Owing to the small number of events no statistical analysis was performed. The remaining metastatic lesions displaying FasL expression developed from $FasL^-$ primaries. Still, although serial sections of primary tumors were performed we cannot exclude that parts of the tumor expressing FasL were missed. This notion is particularly important if the expression pattern of FasL in the positive primary tumors is taken into account.

To establish the relative frequency of FasL expression on metastatic melanoma 17 additional cutaneous metastases were characterized regarding FasL expression. FasL was present at various degrees in nine of these 17 tumors; thus, 54% of the lesions were FasL positive (**Table II**). The summarized data of the two series indicate that the FasL are expressed in approximately 50% of melanoma lesions metastatic to the skin.

FasL expression is associated with the occurrence of apoptotic cells *in situ* It has been suggested that FasL expression might induce apoptosis in Fas-expressing tumor infiltrating lymphocytes; thus, FasL expression would contribute to the immune escape of melanoma. In order to test this hypothesis we analyzed all metastatic tumors from the first series for the presence of apoptotic cells using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling technique. With this approach apoptotic cells could be detected in seven of 11 tumors (**Table I**). Six of these also displayed FasL expression on the melanoma cells. The number of apoptotic cells, however, never exceeded 5%. It should be emphasized that the technique used does not disclose the nature of these apoptotic cells. Hence, it is not possible to formally prove whether the apoptotic cells depict tumor infiltrating lymphocytes or melanoma cells, even if the morphologic appearance of the apoptotic cells suggest their lymphatic origin (**Fig 1*c***) and serial sections stained with an anti-CD3 antibody demonstrated that the apoptotic cells were localized in the same areas where $CD3^+$ cells accumulated (**Fig 1*d***).

DISCUSSION

Recently, Fas/FasL interactions have been accounted for the immune-privileged state of the eye, testis, and placenta. Furthermore, the expression of FasL on different tumors including melanoma have been reported, suggesting a novel immune escape mechanism. As FasL is absent on normal melanocytes, it is supposed to be upregulated during tumorigenesis or melanoma progression (Hahne *et al*, 1996). Thus, the present study was designed to determine FasL expression in primary melanoma and subsequently evolving metastases. In primary tumors FasL expression was only present in about 10%, while in metastatic lesions FasL was present in more than 50%. The latter was confirmed by the detection of FasL in a second series of experiments which comprises solely metastatic tumors. It should be stressed that only skin metastases were analyzed. Thus, the expression of FasL seems to be linked to the stage of disease with predominant expression in metastatic lesions. In order to analyse the functional relevance of FasL expression we tested for the presence of apoptotic cells within metastatic tumors. This analysis revealed a significant, but low (less than 5%) number of such cells. The origin of these cells remains

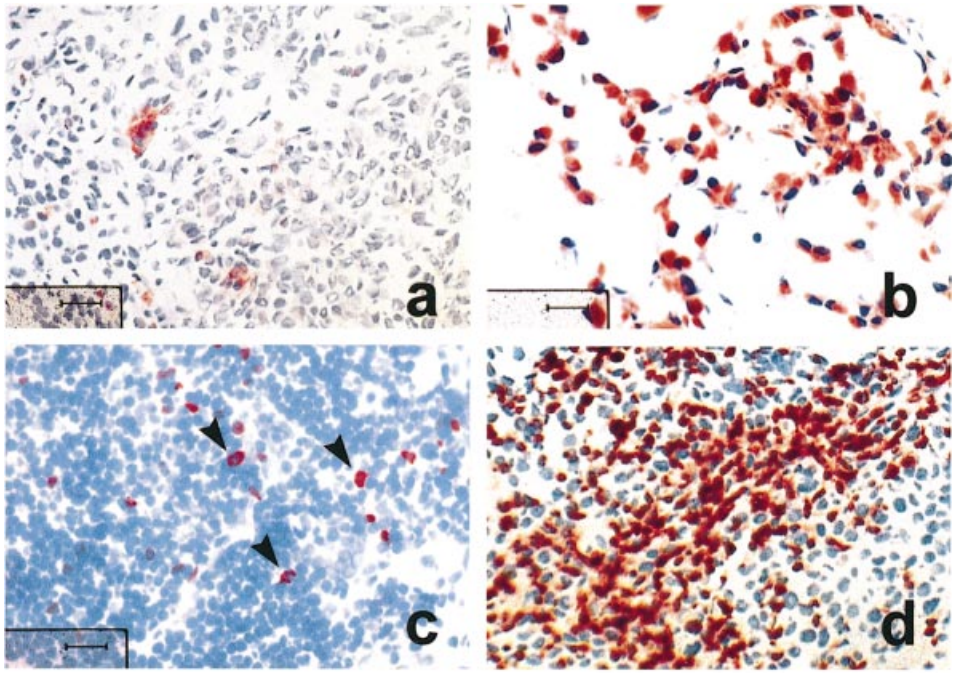

Figure 1. Detection of FasL expression and apoptosis in melanoma. Immunohistology staining with an anti-FasL monoclonal antibody of a primary melanoma (*a*) and of a subsequently evolving metastasis (*b*); representative sections are shown. *In situ* detection of apoptotic cells in a metastasis detected by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling as described under *Materials and Methods* (*c*). Exemplary cells that were assessed as apoptotic are indicated by *arrowheads*. A subsequent section of this lesion was subjected to immunostaining with an anti-CD3 antibody (*d*). Sections were counterstained with hematoxylin. *Scale bar*: 30 μm.

Table II. Expression of FasL in metastatic lesions (nonlongitudinal analysis)

Age (y)	Gender	FasL expression	
		Frequency[a]	Intensity[b]
80	male	Ø	
76	female	Ø	
75	male	Ø	
73	male	+++	+++
73	female	+	++
70	male	+	+
66	male	+	+++
66	male	Ø	
64	female	+	+
64	female	Ø	
62	female	+	++
61	female	Ø	
60	male	++	+
53	male	+	++
45	male	+++	+++
41	female	Ø	
21	female	Ø	

[a]Ø, no; +, less than 5%; ++, between 5 and 50%; +++, more than 50% positive cells.
[b]+, dim; ++, intermediate; +++, bright.

elusive, but morphologic features and their localization in areas of CD3 expression are highly suggestive for them being tumor infiltrating lymphocytes.

In a recent report Benett *et al* (1998) describe a variable extent of FasL expression in primary esophageal carcinomas which correlated significantly to the occurrence of apoptotic cells expressing CD45. Human melanoma cells express FasL and induce apoptosis in infiltrating T cells (Hahne *et al*, 1996). Our present results do confirm these findings. Neither their nor our data, however, exclude that other Fas-expressing cells, i.e., granulocytes, natural killer cells, or melanoma cells themselves contribute to the observed pool of apoptotic cells within the tumor (Liles *et al*, 1996). Thus, both early nonspecific immune responses as well as specific T cell mediated could be inhibited by Fas/FasL interactions. Interestingly, it was shown that the expression of cellular $FLIP_L$ (FLICE-inhibitory protein, long form) by Fas^+ melanoma cells render these cells nonsusceptible to FasL-induced signals; hence, prohibiting autocrine or paracrine suicide (Irmler *et al*, 1997).

It should be noted that T cells need to be susceptible for the FasL-mediated apoptosis. The susceptibility of T cells is dependent on the stage of cell activation, with resting and early activated, i.e., 1–3 d after stimulation, T cells being resistant to Fas-induced apoptosis, whereas the majority of days 4–6-activated T cells undergo apoptosis after cross-linking of Fas (Alderson *et al*, 1995). Rivoltini *et al* (1998) demonstrated that melanoma-reactive $CD4^+$ and $CD8^+$ T cell clones were not influenced by FasL expression of their corresponding melanoma targets as well as soluble FasL or $FasL^+$ lymphocytes. The fact that these antigen-specific T cells are resistant to FasL-induced apoptosis suggests that the proposed immune escape mechanism is not an universal phenomenon. Our data and that of previous studies, however, indicate that apoptosis occurs in melanoma-infiltrating lymphocytes even if it is not prominent or restricted to only susceptible cells.

FasL expression of melanoma cell lines has been correlated with tumorigenicity in an experimental model. Transfer of $FasL^+$ B16-F10 melanoma cells into syngeneic wild-type as well as FasL-deficient *gld* mice led to increased tumorigenicity while the injection of cells into *lpr*, i.e., Fas-negative, mice led to reduced tumor growth (Hahne *et al*, 1996). In our study we observed an increased expression of FasL in cutaneous metastases as compared with their progenitor primaries and FasL expression being restricted to thick tumors; thus, FasL expression is likely to correlate with tumorigenicity of melanoma cells. A recent nonlongitudinal study demonstrating the expression of FasL on advanced primary melanoma and lymph node metastases but not benign melanocytic lesions corroborates with this hypothesis (Maeda *et al*, 1998).

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